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(57) Abstract

There are provided variants of a parent *Pseudomonas* lipase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved. In particular, the compatibility to anionic surfactants has been improved by reducing the binding of anionic surfactants to the enzyme.

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MODIFIED PSEUDOMONAS LIPASES AND THEIR USE

TECHNICAL FIELD

The present invention generally relates to the field of lipolytic enzymes. More in particular, the invention is concerned with Pseudomonas lipases which have been modified by means of recombinant DNA techniques, with methods for their production and with their use, particularly in enzymatic detergent compositions.

BACKGROUND AND PRIOR ART

Lipolytic enzymes are enzymes which are capable of hydrolysing triglycerides into free fatty acids and diglycerides, monoglycerides and eventually glycerol. Some lipolytic enzymes can also split more complex esters such as cutin layers in plants or sebum of the skin. Lipolytic enzymes are used in industry for various enzymatic processes such as the inter- and trans-esterification of triglycerides and the synthesis of esters. They are also used in detergent compositions with the aim to improve the fat-removing properties of the detergent product.

The most widely used lipolytic enzymes for detergents use are lipases (EC 3.1.1.3). For example, EP-A-258 068 and EP-A-305 216 (both Novo Nordisk) describe the production of fungal lipases via heterologous host micro-organisms by means of rDNA techniques, especially the lipase from Thermomyces lanuginosus/Humicola lanuginosa. EP-A-331 376 (Amano) describes a lipase from Pseudomonas cepacia and its production by means of rDNA techniques. Further examples of lipases produced by rDNA technique are given in WO-A-89/09263 and EP-A-218 272 (both Gist-Brocades). It has also been reported that Pseudomonas lipases require a "modulator" or "helper" protein in order to achieve expression of the lipase gene, see WO-A-94/02617 (Gist-Brocades), WO-A-91/00908 (Novo Nordisk) and EP-A-464 922 (Unilever).

In spite of the large number of publications on lipases and their modifications, only the lipase from Humicola lanuginosa has so far found wide-spread commercial

application as additive for detergent products under the trade name Lipolase (TM).

A characteristic feature of lipases is that they exhibit interfacial activation. This means that the enzyme activity is much higher on a substrate which has formed interfaces or micelles, than on fully dissolved substrate. Interface activation is reflected in a sudden increase in lipolytic activity when the substrate concentration is raised above the critical micel concentration (CMC) of the substrate, and interfaces are formed. Experimentally, this phenomenon can be observed as a discontinuity in the graph of enzyme activity versus substrate concentration.

The mechanism of interfacial activation in lipases has been interpreted in terms of a conformational change in the protein structure of the lipase molecule. In the free, unbound state, a helical lid covers the catalytic binding site. Upon binding to the lipid substrate, the lid is displaced and the catalytic site is exposed. The helical lid is also believed to interact with the lipid interface, thus allowing the enzyme to remain bound to the interface.

WO-A-92/05249 (Novo Nordisk) discloses genetically modified lipases, in particular the lipase from Humicola lanuginosa, which have been modified at the lipid contact zone. The lipid contact zone is defined in the application as the surface which in the active form is covered by the helical lid. The modifications involve deletion or substitution of one or more amino acid residues in the lipid contact zone, so as to increase the electrostatic charge and/or decrease the hydrophobicity of the lipid contact zone, or so as to change the surface conformation of the lipid contact zone. This is achieved by deleting one or more negatively charged amino acid residues in the lipid contact zone, or substituting these residues by neutral or more positively charged amino acids, and/or by substituting one or more neutral amino acid residues in the lipid contact zone by positively charged amino acids, and/or deleting one or more hydrophillic amino acid residues in the lipid contact zone, or substituting these residues by hydrophobic amino acids.

In EP-A-407 225 (Unilever) various Pseudomonas lipase variants are disclosed. The lipase variants are stated to provide improved performance in use, e.g. by improved stability against attack by protease and/or oxidising agents and or increased activity by comparison with the parent enzyme. One of the many lipase variants is a variant of the Pseudomonas glumae lipase, in which His Arg 8 has been replaced with Asp (R8D). No further data of this variant are shown. The publication provides no indication how lipase variants may be obtained having improved compatibility to anionic surfactants.

The cutinases (EC 3.1.1.50) are another class of lipolytic enzymes. These enzymes are capable of degrading cutin, a network of esterified long-chain fatty acids and fatty alcohols which occurs in plants as a protective coating on leaves and stems. In addition, they possess some lipolytic activity, i.e. they are capable of hydrolysing triglycerides. Thus they can be regarded as a special kind of lipases. Contrary to lipases, however, cutinases do not exhibit any substantial interfacial activation.

Cutinases can be obtained from a number of sources, such as plants (e.g. pollen), bacteria and fungi. Because of their fat degrading properties, cutinases have been proposed as ingredients for enzymatic detergent compositions. For example, WO-A-88/09367 (Genencor) suggests combinations of a surfactant and a substantially pure bacterial cutinase enzyme to formulate effective cleaning compositions. Disclosed are detergent compositions comprising a cutinase obtained from the Gram negative bacterium Pseudomonas putida ATCC 53552. However, in the more recent European patent application EP-A-476 915 (Clorox), it is disclosed that the same enzyme - which is then referred to as a lipase - is no more effective than other lipases in removing oil stains from fabrics, when used by conventional methods.

Recently, the three-dimensional structure has been determined of a cutinase from Fusarium solani pisi (Martinez et al. (1992) Nature 356, 615-618). It was found that this cutinase does not possess a helical lid to cover the

catalytic binding site. Instead, the active site serine residue appears to be accessible to the solvent. These findings appear to confirm the present theory about the mechanism of interfacial activation in lipases.

The cutinase gene from Fusarium solani pisi has been cloned and sequenced (Ettinger et al., (1987) Biochemistry 26, 7883-7892). WO-A-90/09446 (Plant Genetics Systems) describes the cloning and production of this gene in E. coli. The cutinase can efficiently catalyse the hydrolysis and the synthesis of esters in aqueous and non-aqueous media, both in the absence and the presence of and interface between the cutinase and the substrate. On the basis of its general stability, it is suggested that this cutinase could be used to produce cleaning agents such as laundry detergents and other specialized fat dissolving preparations such as cosmetic compositions and shampoos. A way to produce the enzyme in an economic feasible way is not disclosed, neither are specific enzymatic detergent compositions containing the cutinase.

As mentioned above, only the lipase derived from Humicola lanuginosa has so far found wide-spread commercial application as additive for detergent products under the trade name Lipolase (TM). In his article in Chemistry and Industry 1990, pages 183-186, Henrik Malmos notes that it is known that generally the activity of lipases during the washing process is low, and Lipolase (TM) is no exception. During the drying process, when the water content of the fabric is reduced, the enzyme regains its activity and the fatty stains are hydrolysed. During the following wash cycle the hydrolysed material is removed. This also explains why the effect of lipases is low after the first washing cycle, but significant in the following cycles. Thus, there is still a need for lipolytic enzymes which exhibit any significant activity during the washing process.

In WO-A-94/03578 (Unilever) it is disclosed that certain lipolytic enzymes, in particular cutinase from Fusarium solani pisi, exhibit a clear in-the-wash effect. However, there is still a need for other lipolytic enzymes

having improved properties, such as in-the-wash lipolytic activity and compatibility to anionic surfactants, and for methods for producing such enzymes.

The purpose of the present invention is to provide novel lipolytic enzymes, which have been modified so as to improve their performance, especially their compatibility to anionic surfactants.

We have now surprisingly found that the compatibility of Pseudomonas lipases to anionic surfactants can be improved. In accordance with the present invention, there are provided lipase variants of a parent Pseudomonas lipase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved. More in particular, it was found that the lipolytic activity of Pseudomonas lipases, especially of lipases from Pseudomonas glumae or Pseudomonas pseudoalcaligenes, in anionic-rich detergent compositions may be improved by reducing the binding of anionic surfactants to the enzyme.

DEFINITION OF THE INVENTION

A lipase variant of a parent Pseudomonas lipase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved, in particular by reducing the binding of anionic surfactants to the enzyme.

DESCRIPTION OF THE INVENTION

The invention relates to variants of Pseudomonas lipase enzymes. As discussed above, Pseudomonas lipases an be obtained from a number of Pseudomonas sources. The lipase to be used as parent Pseudomonas lipase or starting material in the present invention for the modification by means of recombinant DNA techniques, is preferably chosen from Pseudomonas glumae or Pseudomonas pseudoalcaligenes. The production of lipase from Pseudomonas glumae is described in

EP-A-407 225 and EP-A-464 922 (both Unilever) and the production of Pseudomonas pseudoalcaligenes lipase is disclosed in EP-A-334 462 and WO-A-94/02617 (both Gist-Brocades). When used in certain detergent compositions, these Pseudomonas lipases may exhibit some "in-the-wash" effects.

Also suitable as parent Pseudomonas lipase or starting material in the present invention for the modification by means of recombinant DNA techniques, are Pseudomonas lipases having a high degree of homology of their amino acid sequence to the lipase from Pseudomonas glumae or Pseudomonas pseudoalcaligenes.

Alternative to the improvement of Pseudomonas glumae or Pseudomonas pseudoalcaligenes lipases by modification of its gene, genetic information encoding lipases from other organisms can be isolated using 5'- and 3'- DNA probes derived from Pseudomonas glumae or Pseudomonas pseudoalcaligenes cDNA encoding (pro)lipase and probes recognizing conserved sequences in other lipolytic enzymes. These probes can be used to multiply cDNA's derived from messenger RNA's (mRNA's) of lipase producing organisms using the Polymerase Chain Reaction or PCR technology (see, for example WO-A-92/05249). In this way a number of natural occurring variants of the above mentioned lipases can be obtained with improved in-the-wash performance. Moreover, the sequences of these natural occurring lipases provide an excellent basis for further protein engineering of the lipases from Pseudomonas glumae or Pseudomonas pseudoalcaligenes.

On the basis of new ideas about the factors determining the activity of "in-the-wash" lipolytic enzymes and careful inspection of the 3D structure of Pseudomonas glumae lipase we have found a number of possibilities how to improve the compatibility of this lipase and Pseudomonas lipases in general to anionic surfactants by means of recombinant DNA techniques.

Starting from the known 3D structure of the Pseudomonas glumae lipase (Noble et al., 1993), the 3D-structure of the lipase from Pseudomonas pseudoalcaligenes

was obtained by applying rule-based comparative modelling techniques as implemented in the COMPOSER module of the SYBYL molecular modelling software package (TRIPOS associates, Inc. St. Louis, Missouri). The obtained model of the Pseudomonas pseudoalcaligenes lipase was refined by applying energy minimization (EM) and molecular dynamics (MD) techniques as implemented in the BIOSYM molecular modelling software package (BIOSYM, San Diego, California). During EM and MD refinement of the model a knowledge-based approach was applied. The model was simultaneously optimized for the detailed energy terms of the potential energy function and known structural criteria. Model quality was assessed by criteria such as number and quality of hydrogen bonds, hydrogen bonding patterns in the secondary structure elements, the orientation of peptide units, the values of and main chain dihedral angles, the angle of interaction of aromatic groups and the sizes of cavities. Moreover, the model was checked for inappropriately buried charges, extremely exposed hydrophobic residues and energetically unfavourable positions of disulphide bridges. Relevant side-chain rotamers were selected from the Ponder & Richards rotamer library (Ponder et al. (1987) J.Mol.Biol. 193, 775-791). The final choice of a particular side-chain rotamer from this library was based on structural criteria evaluations as mentioned above. MD was used to anneal the side-chain atoms into position.

The present invention shows that Pseudomonas lipases can be modified in such a way that the interaction with anionic surfactants can be reduced without destroying the "in-the-wash" performance of the modified Pseudomonas lipase.

This may be achieved in a number of ways. First, the binding of anionic surfactants to the enzyme may reduced by reducing the electrostatic interaction between the anionic surfactant and the enzyme. For instance, by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of an anionic surfactant, by lysine residues. It is also

possible to reduce the electrostatic interaction between the anionic surfactant and the enzyme shielding the positive charge of such an arginine residue by introducing within a distance of about 6 Å from said arginine a negative charge, e.g. an glutamic acid residue. Alternatively, the electrostatic interaction between the anionic surfactant and the enzyme may be reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by uncharged amino acid residues. Furthermore, the electrostatic interaction between the anionic surfactant and the enzyme may reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by negatively charged amino acid residues.

Another approach to reduce the binding between an anionic surfactant and the enzyme is to replace one or more amino acid residues which are located in a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by less hydrophobic amino acid residues. These less hydrophobic amino acid residues are preferably selected from the group consisting of glycine, serine, alanine, aspartic acid and threonine.

Due to their improved anionics compatibility, the lipase variants produced according to the invention can bring advantage in enzyme activity, when used as part of an anionic-rich detergent or cleaning compositions. In the context of this invention, anionic-rich means that the detergent or cleaning composition contains a surfactant system which consists for more than 5%, generally more than 10%, and in particular more than 20% of anionic surfactants.

The Pseudomonas lipase variants of the present invention were found to possess a certain in-the-wash performance during the main cycle of a wash process. By in-the-wash performance during the main cycle of a wash process, it is meant that a detergent composition containing the enzyme is capable of removing a significant amount of oily

soil from a soiled fabric in a single wash process in a European type of automatic washing machine, using normal washing conditions as far as concentration, water hardness, temperature, are concerned. It should be born in mind that under the same conditions, the conventional commercially available lipolytic enzyme Lipolase (TM) ex Novo Nordisk does not appear to have any significant in-the-wash effect at all on oily soil.

The in-the-wash effect of an enzyme on oily soil can be assessed using the following assay. New polyester test having a cotton content of less than 10% are prewashed using an enzyme-free detergent product such as the one given below, and are subsequently thoroughly rinsed. Such unsoiled cloths are then soiled with olive oil or another suitable, hydrolysable oily stain. Each tests cloth (weighing approximately 1 g) is incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The wash liquor contains the detergent product given below at a dosage of 1 g per litre. The bottles are agitated for 30 minutes in a Miele TMT washing machine filled with water and using a normal 30°C main wash programme. The lipase variant is preadded to the wash liquor at 3 LU/ml. The control does not contain any enzyme. The washing powder has the following composition (in % by weight):

TABLE I

LAS	6.9
Soap	2.0
Nonionic surfactant	10.0
Zeolite	27.0
Sodium carbonate	10.2
Sodium sulphate	13.0

After washing, the cloths are thoroughly rinsed with cold water and dried in a tumble dryer with cold air, and the amount of residual fat is assessed. This can be done in several ways. The common method is to extract the testcloth with petroleum ether in a Soxhlet extraction apparatus, distilling off the solvent and determining the

percentage residual fatty material as a fraction of the initial amount of fat on the cloth by weighing.

According to a second, more sensitive method, brominated olive oil is used to soil the test cloths (Richards, S., Morris, M.A. and Arklay, T.H. (1968), Textile Research Journal 38, 105-107). Each test cloth is then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. A series of bottles is then agitated in a washing machine filled with water and using a normal 30°C main wash programme. After the main wash, the test cloths are carefully rinsed in cold water during 5 seconds. Immediately after the rinse, the test cloths dried in a dryer with cold air. After drying the amount of residual fat can be determined by measuring the bromine content of the cloth by means of X-ray fluorescence spectrometry. The fat removal can be determined as a percentage of the amount which was initially present on the test cloth, as follows:

$$\%\ \text{Soil removal} = \frac{\text{Bromine}_{bw} - \text{Bromine}_{aw}}{\text{Bromine}_{bw}} * 100\ \%$$

wherein: Bromine_{bw} denotes the percentage bromine on the cloth before the wash and Bromine_{aw} the percentage bromine after the wash.

A further method of assessing the enzymatic activity is by measuring the reflectance at 460 nm according to standard techniques.

In the context of this invention, a modified, mutated or mutant enzyme or a variant of an enzyme means an enzyme that has been produced by a mutant organism which is expressing a mutant gene. A mutant gene (other than one containing only silent mutations) means a gene encoding an enzyme having an amino acid sequence which has been derived directly or indirectly, and which in one or more locations is different, from the sequence of a corresponding parent enzyme. The parent enzyme means the gene product of the corresponding unaltered gene. A silent mutation in a gene means a change or difference produced in the polynucleotide sequence of the gene which (owing to the redundancy in the

codon-amino acid relationships) leads to no change in the amino acid sequence of the enzyme encoded by that gene.

A mutant or mutated micro-organism means a micro-organism that is, or is descended from, a parent micro-organism subjected to mutation in respect of its gene for the enzyme. Such mutation of the organism may be carried out either (a) by mutation of a corresponding gene (parent gene) already present in the parent micro-organism, or (b) by the transfer (introduction) of a corresponding gene obtained directly or indirectly from another source, and then introduced (including the mutation of the gene) into the micro-organism which is to become the mutant micro-organism. A host micro-organism is a micro-organism of which a mutant gene, or a transferred gene of other origin, forms part. In general it may be of the same or different strain or species origin or descent as the parent micro-organism.

In particular, the invention provides mutant forms of the <u>Pseudomonas glumae</u> lipase and of the <u>Pseudomonas pseudoalcaligenes</u> lipase. These lipase variants can be produced by a rDNA modified micro-organism containing a gene obtained or made by means of rDNA techniques.

It will be clear to the skilled man that such modifications will affect the structure of the <u>Pseudomonas</u> lipase. Obviously, modifications are preferred which do not affect the electrostatic charge around the active site too much. The inventors have developed the necessary level of understanding of the balance between the inevitable distortion of the conformation of the enzyme and the benefit in increased enzyme activity, which makes is possible to predict and produce successful lipase variants with a high rate of success. In the following Table II and elsewhere in this specification, amino-acids and amino acid residues in peptide sequences are indicated by one-letter and three-letter abbreviations as follows:

<u>TABLE II</u>

A	=	Ala	=	Alanine	V	=	Val	=	Valine
L	=	Leu	=	Leucine	I	=	Ile	=	Isoleucine
P	=	Pro	=	Proline	F	=	Phe	=	Phenylalanine

W	=	Trp	=	Tryptophan	M	=	Met	=	Methionine
G	=	Gly	=	Glycine	S	=	Ser	=	Serine
T	=	Thr	=	Threonine	C	=	Cys	=	Cysteine
Y	=	Tyr	=	Tyrosine	N	=	Asn	=	Asparagine
Q	=	Gln	=	Glutamine	D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid	K	=	Lys	=	Lysine
R	=	Arg	=	Arginine	H	=	His	=	Histidine

In this specification, a mutation present in the amino acid sequence of a protein, and hence the mutant protein itself, may be described by the position and nature of the mutation in the following abbreviated way: by the identity of an original amino acid residue affected by the mutation; the site (by sequence number) of the mutation; and by the identity of the amino acid residue substituted there in place of the original. If there is an insertion of an extra amino acid into the sequence, its position is indicated by one or more subscript letters attached to the number of the last preceding member of the regular sequence or reference sequence.

For example, a mutant characterised by substitution of Arginine by Glutamine in position 17 is designated as: Arg17Glu or R17E. A (hypothetical) insertion of an additional amino acid residue such as proline after the Arginine would be indicated as Arg17ArgPro or R17RP, alternatively as *17aP, with the inserted residue designated as position number 17a. A (hypothetical) deletion of Arginine in the same position would be indicated by Arg17* or R17*. The asterisk stands either for a deletion or for a missing amino acid residue in the position designated, whether it is reckoned as missing by actual deletion or merely by comparison or homology with another or a reference sequence having a residue in that position.

Multiple mutations are separated by plus signs, e.g. R17E+S54I+A128F designates a mutant protein carrying three mutations by substitution, as indicated for each of the three mentioned positions in the amino acid sequence. The mutations given in the following Table may be combined if desired.

Table III below shows certain useful examples of Pseudomonas lipase variants according to the invention, based on the sequence of the lipase from Pseudomonas glumae and from Pseudomonas pseudoalcaligenes.

TABLE III

Variants of Pseudomonas glumae lipase:
Y4D, R8D, K22D, K43D, K70E, R165E, R177D, Y196D, T244E, I277E, R296D, A298D, L316E.

Variants of Pseudomonas pseudoalcaligenes lipase:
Y8D, K12D, R41N, K195D, I247E, L286E.

Using his general knowledge and the information available in WO-A-94/03578 (Unilever), EP-A-464 922 (Unilever) and EP-A-334 462 (Gist-Brocades), the skilled man will have no difficulty in preparing other variants of Pseudomonas lipases, or variants from Pseudomonas lipases from other sources.

According to a further aspect of the invention, there is provided a process for producing the lipase variants of the invention. Because Pseudomonas organisms often require a "modulator" or "helper" protein in order to achieve expression of the lipase gene, the production is primarilty carried out using a suitable Pseudomonas strain as host organism. For that purpose, the genes coding for modified (pro)lipases may be integrated in rDNA vectors that can be transferred into other host micro-organisms by means of rDNA technology. For this purpose rDNA vectors essentially similar to the rDNA vector described in EP-A-407 225 can be used.

The invention also provides genetic material derived from the introduction of modified Pseudomonas lipase genes, e.g. the gene from Pseudomonas pseudoalcaligenes into cloning rDNA vectors, and the use of these to transform new host cells and to express the genes of the lipase variants in the new host cells.

Also provided by the invention are polynucleotides made or modified by rDNA technique, which encode such lipase variants, rDNA vectors containing such polynucleotides, and

rDNA modified micro-organisms containing such polynucleotides and/or such rDNA vectors. The invention also provides corresponding polynucleotides encoding the modified Pseudomonas lipases, e.g. a polynucleotide having a base sequence that encodes a mature lipase variant, in which polynucleotide the final translated codon is followed by a stop codon and optionally having nucleotide sequences coding for the prepro- or pro-sequence of this lipase variant directly upstream of the nucleotide sequences coding for the mature lipase variant.

In such a polynucleotide, the lipase-encoding nucleotide sequence derived from the organism of origin can be modified in such a way that at least one codon, and preferably as many codons as possible, are made the subject of 'silent' mutations to form codons encoding equivalent amino acid residues and being codons preferred by a new host, thereby to provide in use within the cells of such host a messenger-RNA for the introduced gene of improved stability.

Upstream of the nucleotide sequences coding for the pro-or mature lipases, there can be located a nucleotide sequence that codes for a signal or secretion sequence suitable for the chosen host. Thus an embodiment of the invention relates to a rDNA vector into which a nucleotide sequence coding for a lipase variant or a precursor thereof has been inserted.

The nucleotide sequence can be derived for example from:

(a) a naturally occurring nucleotide sequence (e.g. encoding the original amino acid sequence of lipase produced by Pseudomonas pseudoalcaligenes);

(b) chemically synthesized nucleotide sequences consisting of codons that are preferred by the new host and a nucleotide sequence resulting in stable messenger RNA in the new host, still encoding the original amino acid sequence;

(c) genetically engineered nucleotide sequences derived from one of the nucleotide sequences mentioned in preceding paragraphs a or b coding for a Pseudomonas pseudoalcaligenes

lipase with a different amino acid sequence but having superior stability and/or activity in detergent systems.

Summarizing, rDNA vectors able to direct the expression of the nucleotide sequence encoding a Pseudomonas lipase gene as described above in one of the preferred hosts preferably comprise the following components:

(a) Double-stranded (ds) DNA coding for mature Pseudomonas lipase or prelipase or a corresponding prelipase in which at least part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell). In cases where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front. The translated part of the gene should always end with an appropriate stop codon;

(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the Pseudomonas lipase (component (a));

(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the Pseudomonas lipase (component (a);

(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,

(d2) an origin of replication suitable for the selected host;

(e1) Optionally a (auxotrophic) selection marker. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter;

(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the lipase in the host selected.

Such a rDNA vector can also carry, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the lipase. The auxotrophic marker can consist of a coding region of the auxotrophic marker and a defective promoter region.

Another embodiment of this invention is the fermentative production of one of the various Pseudomonas lipase variants described above. Such a fermentation can either be a normal batch fermentation, fed-batch fermentation

or continuous fermentation. The selection of a process to be used depends on the host strain and the preferred down stream processing method (known per se). Thus, the invention also provides a process for producing a Pseudomonas lipase variant as specified herein, which comprises the steps of fermentatively cultivating an rDNA modified micro-organism containing a gene made by rDNA technique which carries at least one mutation affecting the amino acid sequence of the lipase thereby to confer upon the Pseudomonas lipase improved activity by comparison with the corresponding parent enzyme, making a preparation of the Pseudomonas lipase variant by separating the lipase produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the lipase variant either from said broth or from said cells by physical or chemical concentration or purification methods. Preferably conditions are chosen such that the Pseudomonas lipase variant is secreted by the micro-organism into the fermentation broth, the enzyme being recovered from the broth after removal of the cells either by filtration or centrifugation. Optionally, the Pseudomonas lipase variant can then be concentrated and purified to a desired extent. These fermentation processes in themselves apart from the special nature of the micro-organisms can be based on known fermentation techniques and commonly used fermentation and down stream processing equipment.

Also provided by the invention is a method for the production of a modified micro-organism capable of producing a Pseudomonas lipase variant by means of rDNA techniques, characterized in that the gene coding for the lipase variant that is introduced into the micro-organism is fused at its 5'-end to a gene fragment encoding a (modified) pre-sequence functional as a signal- or secretion-sequence for the host organism.

According to a further aspect of the invention, there are provided rDNA modified micro-organisms containing a Pseudomonas lipase variant gene and able to produce the

lipase variant encoded by said gene. In an rDNA modified micro-organism, a gene (if originally present) encoding the native lipase is preferably removed, e.g. replaced by another structural gene.

According to a further aspect of the present invention, there are provided enzymatic detergent compositions comprising the Pseudomonas lipase variants of the invention. Such compositions are combinations of the Pseudomonas lipase variants and other ingredients which are commonly used in detergent systems, including additives for detergent compositions and fully-formulated detergent and cleaning compositions, e.g. of the kinds known per se and described for example in EP-A-258 068. More specifically, they may comprise from 5 - 60%, preferably from 20 - 50% by weight of a detergency builder and from 0.1 - 50 % by weight of an active system, which in turn may comprise 0 - 95 % by weight of one or more anionic surfactants and 5 - 100 % by weight of one or more nonionic surfactants.

The other components of such detergent compositions can be of any of many known kinds, for example as described in GB-A-1 372 034 (Unilever), US-A-3 950 277, US-A-4 011 169, EP-A-179 533 (Procter & Gamble), EP-A-205 208 and EP-A-206 390 (Unilever), JP-A-63-078000 (1988), and Research Disclosure 29056 of June 1988, together with each of the several specifications mentioned therein, all of which are hereby incorporated herein by reference.

The Pseudomonas lipase variants of the present invention can usefully be added to the detergent composition in any suitable form, i.e. the form of a granular composition, a solution or a slurry of the enzyme, or with carrier material (e.g. as in EP-A-258 068 and the Savinase(TM) and Lipolase(TM) products of Novo Nordisk).

The added amount of Pseudomonas lipase variant can be chosen within wide limits, for example from 10 - 20,000 LU per gram, and preferably 50 -2,000 LU per gram of the detergent composition. In this specification LU or lipase units are defined as they are in EP-A-258 068 (Novo Nordisk). Similar considerations apply mutatis mutandis in the case of

other enzymes, which may also be present. Examples of other useful enzymes are, for example, proteases, amylases and cellulases.

Advantage may be gained in such detergent compositions, where protease is present together with the cutinase, by selecting such protease from those having pI lower than 10. EP-A-271 154 (Unilever) describes a number of such proteases. Proteases for use together with Pseudomonas lipase variants can in certain circumstances include subtilisin of for example BPN' type or of many of the types of subtilisin disclosed in the literature, some of which have already been proposed for detergents use, e.g. mutant proteases as described in for example EP-A-130 756 or EP-A-251 446 (both Genentech); US-A-4 760 025 (Genencor); EP-A-214 435 (Henkel); WO-A-87/04661 (Amgen); WO-A-87/05050 (Genex); Thomas et al. (1986/5) Nature 316, 375-376 and (1987) J.Mol.Biol. 193, 803-813; Russel et al. (1987) Nature 328, 496-500.

The invention will now be further illustrated in the following Examples.

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Ettinger, W.F., Thukral, S.K. and Kolattukudy, P.E. (1987). Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi. Biochemistry 26, 7883-7892.

Noble, M.E.M., Cleasby, A., Johnson, L.N., Egmond, M.R., and Frenken, L.G.J. (1993). The crystal structure of triacylglycerol lipase from Pseudomonas glumae reveals a partially redundant catalytic aspartate. FEBS Lett. 331, 123-128.

In the Figures is:

Fig. 1. In-the-wash effect for Pseudomonas glumae lipase and the lipase variant R8D.

EXAMPLE 1

Construction of a synthetic gene encoding Pseudomonas glumae lipase and variants thereof.

A synthetic gene encoding Pseudomonas glumae lipase was constructed essentially according to the cassette method described in EP-A-407 225 (Unilever), and the variants thereof were constructed using the method disclosed in the same publication.

EXAMPLE 2

Determining the In-the-wash activity of Pseudomonas glumae variant R8D.

Test cloths made of polyester were soiled with brominated olive oil. Each tests cloth was then incubated in 30 ml wash liquor in a 100 ml polystyrene bottle. The bottles were agitated in a Miele TMT washing machine filled with water and using a normal 30°C main wash programme. The wash liquor consisted of 0.8 grams per litre (at 27°FH) of washing powders A.

Detergent Product A (% by weight)

Linear Alkylbenzene Sulphonate	12.5
Nonionic surfactant C_{12}-C_{15} alcohol.7 EO	12.5
Sodium Tripolyphosphate	31.25
Sodium Silicate	10.0
Sodium Carbonate	12.5
Sdium Sulphate	21.25

The results are shown in Figure 1. The enhancement of the in-the-wash performance (oily soil removal) of Pseudomonas glumae lipase variant R8D relative to wild-type Pseudomonas glumae lipase at various concentrations is evident. For comparison, the same experiments were also carried out with Lipolase (TM). Under all conditions, the Pseudomonas glumae lipase variant R8D was superior.

CLAIMS

1. A lipase variant of a parent Pseudomonas lipase, wherein the amino acid sequence has been modified in such way that the compatibility to anionic surfactants has been improved, with the proviso, that the lipase variant R8D from Pseudomonas glumae is not claimed.

2. A lipase variant according to Claim 1, in which the compatibility to anionic surfactants has been improved by reducing the binding of anionic surfactants to the enzyme.

3. A lipase variant according to any one of the preceding Claims, in which the binding of anionic surfactants to the enzyme has been reduced by reducing the electrostatic interaction between the anionic surfactant and the enzyme.

4. A lipase variant according to any one of Claims 1-3, wherein the electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by lysine residues.

5. A lipase variant according to any one of Claims 1-3, wherein the electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by uncharged amino acid residues.

6. A lipase variant according to any one of Claims 1-3, wherein the electrostatic interaction between the anionic surfactant and the enzyme is reduced by replacing one or more positively charged arginine residues which are located close to a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by negatively charged amino acid residues.

7. A lipase variant according to any one of Claims 1-3, wherein the binding of the anionic surfactant and the enzyme is reduced by replacing one or more amino acid residues which are located in a hydrophobic patch capable of binding the apolar tail of the anionic surfactant, by less hydrophobic amino acid residues.

8. A lipase variant according to Claim 7, in which the less hydrophobic amino acid residues are selected from the group consisting of glycine, serine, alanine, aspartic acid and threonine.

9. A lipase variant according to any of the preceding Claims, wherein the parent lipase is a Pseudomonas glumae lipase or a Pseudomonas pseudoalcaligenes lipase.

10. A lipase variant according to any one of the preceding Claims, in which the parent enzyme is a lipase which is immunologically cross-reacting with antibodies raised against the lipase from Pseudomonas glumae or Pseudomonas pseudoalcaligenes.

11. A lipase variant according to any one of the preceding Claims, encoded by genes that have extensive homology to the 5'- and/or 3' ends of to the genes encoding lipase from Pseudomonas glumae or Pseudomonas pseudoalcaligenes and/or to conserved sequences in these lipases.

12. A lipase variant according to any one of the preceding Claims, in which the modified residues are located at one or more of the following positions in the amino acid sequence of the Pseudomonas glumae lipase, or the corresponding amino acids of a different lipase: 4, 8, 22, 43, 70, 165, 177, 196, 244, 277, 296, 298, 316.

13. A lipase variant according to any one of the preceding Claims, whereby the modified residues are located in the

hydrophobic patch of the Pseudomonas glumae lipase, or the corresponding amino acids of a different lipase.

14. A lipase variant according to any one of the preceding Claims, which is a variant of Pseudomonas glumae lipase and comprises one or more of the following mutations: Y4D, K22D, K43D, K70E, R165E, R177D, Y196D, T244E, I277E, T296D, A298D, L316E.

15. A lipase variant according to any one of the preceding Claims, which is a variant of Pseudomonas pseudoalcaligenes lipase and comprises one or more of the following mutations: Y8D, K12D, R41N, K195D, I247E, L286E.

16. A process for producing a lipase variant according to any one of the preceding Claims, which comprises the steps of fermentatively cultivating an rDNA modified microorganism containing a gene made by rDNA technique which encodes the lipase variant, making a preparation of the lipase variant by separating the lipase variant produced by the micro-organism either from the fermentation broth, or by separating the cells of the micro-organism from the fermentation broth, disintegrating the separated cells and concentrating or part purifying the lipase either from said broth or from said cells by physical or chemical concentration or purification methods.

17. A rDNA modified micro-organism which has been transformed by a rDNA vector carrying a gene encoding a lipase variant according to any of Claims 1 to 15 and which is thereby able to express said lipase variant.

18. A rDNA modified micro-organism according to Claim 17 carrying a gene encoding a lipase variant that is introduced into the micro-organism by fusion at its 5'-end to a gene fragment encoding a (modified) pre-sequence functional as a signal- or secretion-sequence for the host organism.

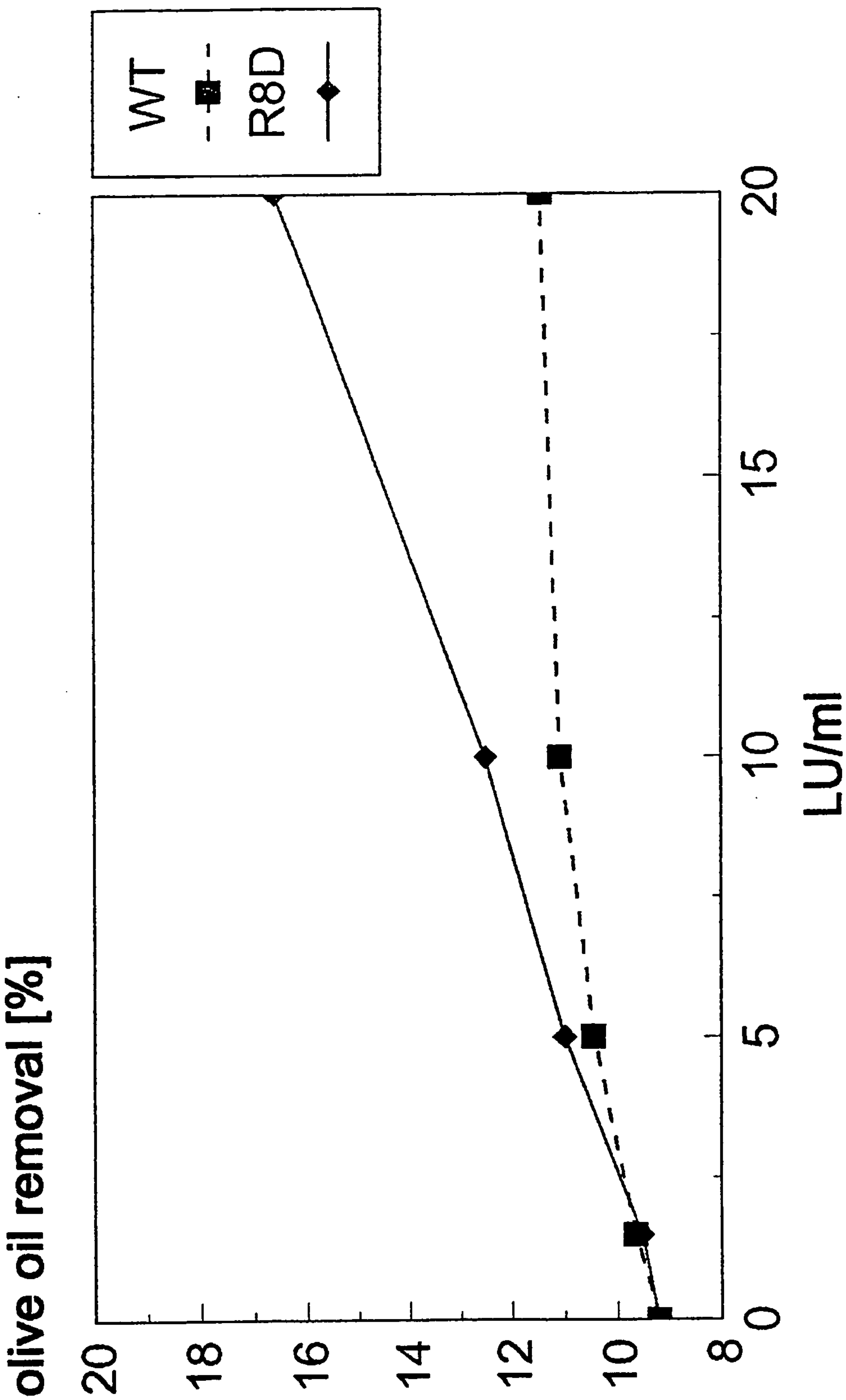
19. A recombinant DNA vector able to direct the expression of a nucleotide sequence encoding a lipase variant gene, comprising the following components:
(a) Double-stranded (ds) DNA coding for the mature lipase variant or prelipase or a corresponding prelipase in which at least part of the presequence has been removed directly down stream of a secretion signal (preferred for the selected host cell), provided that where the part of the gene that should be translated does not start with the codon ATG, an ATG codon should be placed in front, and provided also that the part of the gene to be translated ends with an appropriate stop codon or has such codon added;
(b) An expression regulon (suitable for the selected host organism) situated upstream of the plus strand of the ds DNA encoding the lipase variant (component (a));
(c) A terminator sequence (suitable for the selected host organism) situated down stream of the plus strand of the ds DNA encoding the lipase variant (component (a);
(d1) Nucleotide sequences which facilitate integration, of the ds DNA into the genome of the selected host or,
(d2) an origin of replication suitable for the selected host;
(e1) Optionally a (auxotrophic) selection marker;
(e2) Optionally a ds DNA sequence encoding proteins involved in the maturation and/or secretion of one of the precursor forms of the lipase variant in the host selected.

20. A recombinant DNA vector according to Claim 19, also carrying, upstream and/or downstream of the polynucleotide as earlier defined, further sequences facilitative of functional expression of the lipase.

21. A recombinant DNA vector according to any one of Claims 19 to 20, carrying an auxotrophic marker consisting of a coding region of the auxotrophic marker and a defective promotor region.

28. An enzymatic detergent composition comprising a lipase variant according to any one of Claims 1 to 15.

Fig. 1



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/02350

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N15/78 C12N9/20 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 10695 (OLIN CORPORATION) 20 September 1990 see page 9 - page 10 ---	1-11, 15-22
Y	EP,A,0 464 922 (UNILEVER NV) 8 January 1992 cited in the application see whole document, especially page 4 lines 7-14 ---	1-14, 16-22
P,Y	WO,A,94 14964 (UNILEVER NV) 7 July 1994 see the whole document ---	1-22
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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International Application No
PCT/EP 95/02350

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	EP,A,0 407 225 (UNILEVER PLC) 9 January 1991 cited in the application see the whole document -----	1-22

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